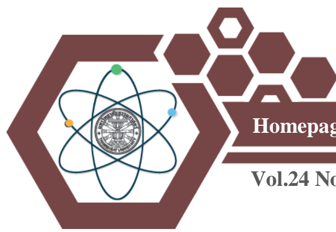

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Original research article

Comparatively Rapid Screening Tests for Diagnosis of Hepatitis B Virus Infection Using Loop-Mediated Isothermal Amplification (LAMP) Paired with Lateral Flow Dipstick (LFD), Gold Nanoparticles (AuNPs) and Real-Time Turbidimetry

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ABSTRACT

Hepatitis B virus (HBV) infects hepatocytes and causes acute and chronic hepatitis that can lead to cirrhosis and hepatocellular carcinoma (HCC) in both animals and humans. Early detection of HBV infection assists in monitoring the patient's response to anti-HBV therapy, blood donation screening, and disease management, control and eradication.

This research focused on development of LAMP assay combined with lateral flow dipstick (LFD), gold nanoparticle (AuNPs) and real-time turbidimetry for screening of the hepatitis B virus. Analytical sensitivity, analytical specificity, diagnostic sensitivity, diagnostic specificity, accuracy and predictive value of each technique were determined and compared to conventional PCR and real-time PCR (gold standard method).

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The analytical sensitivity of LAMP-LFD and LAMP-AuNPs was 1.24×10^1 copies /mL, LAMP-real-time turbidimetry was 1.24×10^2 copies/mL, while that of conventional PCR was 1.24×10^4 copies/mL. Examination of the analytical specificity of all LAMP-based combinations and conventional PCR showed no cross-reactivity with HCV or human plasma. Upon exploration of one hundred unknown samples, in comparison to real-time PCR, the diagnostic sensitivity and specificity of LAMP-based assays were 100% and 90%, respectively. The accuracy, positive predictive value (PPV) and negative predictive value (NPV) of the LAMP-based assays were 98%, 97.56%, and 100%, respectively. While that of conventional PCR were 60%, 100%, 68%, 100% and 38% of diagnostic sensitivity, diagnostic specificity, accuracy, PPV and NPV, respectively.

LAMP-based assays need to be simplified in terms of achieving single-step diagnosis using one master mix solution that is suitable for a point-of-care diagnostic test.

Keywords: Hepatitis B virus (HBV); HBV S gene; Loop-mediated isothermal amplification (LAMP); Lateral flow dipstick (LFD); Gold nanoparticles (AuNPs); Real-time turbidimetry.

1.Introduction

Hepatitis B is a pandemic infectious disease caused by hepatitis B virus (HBV). The disease occurs due to acute and chronic infections affecting the liver and can lead to cirrhosis and hepatocellular carcinoma (HCC) [1]. Acute HBV infection is a common cause of acute icteric hepatitis in adults, although most adults recover and develop lifelong immunity. Chronic HBV infection normally arises beginning in childhood or during birth via contact with infected fluids. Most patients with chronic HBV infection have minimal symptoms and do not develop jaundice after becoming infected with HBV. These patients commonly remain undiagnosed and have immunity against this virus for a long period of time [2].

In many cases, no symptoms can be detected through the incubation period of 40-180 days after HBV infection [3]. Afterwards, acute HBV infection develops during the first 6 months. Later, most healthy people can recover, and the virus is eradicated. In some people, viruses can remain in the cells and blood circulation for over 6 months, possibly leading to chronic HBV infection [1]. An estimated 240 million people are chronically infected with HBV, and more than 686,000 people die every year because of cirrhosis and liver cancer [4]. Hence, rapid and reliable tools for early detection and regular investigation of this virus are key to the management, control and eradication of this disease.

HBV can be transmitted through any mucous membrane of an uninfected person coming into contact with any infected body fluid, including saliva, semen, vaginal secretions, and blood, via various activities such as intravenous drug use, blood transfusion, sexual intercourse and mother-to-child transmission [5, 6]. Nevertheless, the highest concentrations of the virus are found in blood.

Previously, the routine laboratory diagnosis of HBV infection relied on a liver function test, measurement of serology markers, and liver biopsy, which are low sensitivity and time-consuming. Recently, molecular techniques of nucleic acid-based tests (NATs), such as conventional PCR (Polymerase chain reaction) and real-time PCR have been introduced for the detection of HBV. Due to their high sensitivity and specificity, NATs efficiently detect viruses during the window period; however, these techniques require expensive instruments for amplification and PCR product analysis

involves the staining of 2.0% agarose gel electrophoresis (AGE) with carcinogenic ethidium bromide [7].

Thus, the development of LAMP assay paired with alternative detection methods of DNA amplicons could be promoted as a rapid diagnostic test for the direct identification of HBV. In this study, three different detection methods were explored for the detection of DNA amplicons given their convenience of use.

First, the lateral flow test with a lateral flow dipstick (LFD), an easy and convenient nucleic acid-membrane-based chromatography method, was selected for combination with the LAMP assay. LFD has been predominantly used for the immunological detection of several pathogens [8-12]. Modification of the LFD for the discovery of a particular DNA sequence has been achieved in some viruses [13-16]. Initially, the biotin-labeled LAMP product/FITC-labeled DNA probe complex is placed on the sample pad of the LFD membrane containing a rabbit anti-FITC antibody conjugated to gold nanoparticles (AuNPs). The test line of the membrane strip appears as a reddish-colored band representing the biotin ligand/biotin-labeled LAMP product/FITC-labeled DNA probe complex.

Second, gold nanoparticles (AuNPs) can interact with 5'-SH-C6-labeled DNA probes (thiol) via disulfide bonds (S-S). In the presence of a high salt concentration, the non-aggregated DNA/AuNPs complex, present a red color with a localized surface plasmon resonance (LSPR) of 520 nm. In contrast, the aggregation of free AuNPs appears as blue or purple, as indicated by a wavelength shift to 600-700 nm. Generally, NaCl, $MgCl_2$ and $MgSO_4$ are employed to induce the aggregation of free AuNPs. The color change of the reaction can be visualized either by the naked eye or by UV–visible spectral analysis [17-20]. Many previous reports have recommended the DNA/AuNP technique as a simple, convenient, rapid, sensitive and specific method that does not require expensive instruments [21-22].

Lastly, real-time turbidimetry can be performed to detect LAMP products via the formation of magnesium pyrophosphate precipitates [23]. This technique has been introduced for the investigation of several pathogens [24-26]. In principle, DNA polymerization via *Bst* DNA polymerase activity generates pyrophosphate ions from dNTPs, which further interact with magnesium ions in the LAMP reagent to form white precipitates of magnesium pyrophosphate. The reaction time of LAMP/real-time turbidimetry is within 30-60 min. The LAMP products can be subsequently detected by real-time monitoring of amplification using LAMP MEMS software analysis. The data are illustrated as graphs with plots of the turbidity (OD) versus the reaction time (min). The turbidity (OD) of a positive sample ranges from 0.02-1.0, while that of a negative sample appears as linear at zero [27].

Each combination of rapid screening tests for the early detection of HBV (LAMP-LFD, LAMP-AuNPs and LAMP-real-time turbidimetry) was established and evaluated. The analytical sensitivity and specificity, diagnostic sensitivity and specificity, accuracy and predictive value of each technique were determined in comparison to conventional PCR and real-time PCR.

2. Materials and Methods

2.1 Specimens collection and DNA extraction

HBV positive/negative plasma samples and unknown samples were randomly collected from the Her Royal Highness Princess Maha Chakri Sirindhorn Medical Center at Srinakharinwirot

University, Ongkharak, Nakhonnayok, Thailand. The approval of the Human Ethics Committee: Approval No. SWUEC/E-253/2559.

A was confirmed positive and negative samples using the HBsAg serology test and real-time PCR (gold standard method).

DNA extraction was accomplished according to the manufacturer's protocol using QIAamp DNA kits (Qiagen®, Germany). Briefly, DNA was extracted from 200 µL of plasma, eluted in 80-100 µL of Qiagen Buffer AE, and stored at -20°C until use. Aliquots of DNA samples were subjected to HBV screening tests.

2.2 Primers and DNA probes

Sets of PCR and LAMP primers were designed based on the HBV S gene (GenBank accession number: AF068756.1) using Primer Explorer v.4 software (https://primerexplorer.jp/e/) and were aligned using ClustalW software (http://www.genome.jp/tools-bin/clustalw). The forward and reverse primers F3 and B3 were applied for PCR. The LAMP primers consisted of two outer primers; F3 and B3, and two inner primers; FIP and BIP, which recognized a total of six distinct sequences of target DNA. DNA probes were designed based on the region between F1 and F2 in the sequence of the target DNA. Analysis and identification of the primer/probe were attained using NCBI nucleotide BLAST with 100% identity to HBV. The primers and probes were purchased from Pacific Science Co., Ltd. (Biobasic, Canada).

The FIP primer and DNA probe for the LAMP-LFD assay were labeled with biotin and FITC at the 5' ends, respectively. For the LAMP-AuNPs assay, the DNA probe was labeled with a thiol group at the 5' end. Unfortunately, the detail of primers and DNA probes cannot be shown due to a confidential patent (petty patent (number 13136).

2.3 Conventional PCR amplification

Conventional PCR amplification was conducted in a 25µL reaction mixture containing sterile water, 10x PCR buffer (Vivantis, USA), 1.5 µM F3 and B3 primer, 3 mM $MgCl_2$ (Vivantis, USA), 2 mM dNTPs (New England Biolabs, USA), 2 U *Taq* DNA polymerase (Vivantis, USA) and DNA template. Distilled water was used as a blank negative control.

The conventional PCR reaction was performed using a thermal cycler (T100 Thermal Cycler, Bio-Rad, USA). The reaction protocol was 5 min at 95°C for the initial denaturation step, followed by 34 cycles of 30 sec at 95°C for the denaturation step, 30 seconds at 52-58°C for the annealing step, and 30 seconds at 72°C for the extension step. The post-extension step was performed at 72ºC for 5 min. The PCR products were analyzed via 2.0% agarose gel electrophoresis (AGE) at 100 volts for 30 min prior to staining with ethidium bromide and observation under UV light (Gel documentation: Syngene bio-imaging systems G-box).

2.4 LAMP amplification

The LAMP reaction was performed in 25 µL of reaction mixture containing sterile water, 1X supplied buffer (New England Biolabs, USA), 2.0 µM each of FIP and BIP, 0.2 µM each of F3 and B3, 1.6 mM dNTPs (New England Biolabs, USA), 0.5 M betaine (Sigma-Aldrich, USA), variation of $MgSO_4$ concentrations at 1.6-8.0 mM (New England Biolabs, USA), 8 U *Bst* DNA polymerase (Large fragment; New England Biolabs, USA) and DNA template. Distilled water was used as a blank negative control. The reaction mixture was incubated under various temperatures at 60-68°C for 1 hr in a heat block. The LAMP products were further analyzed via 2.0% AGE at 100 volts for 30 min prior to staining with ethidium bromide and observation under

UV light (Gel documentation: Syngene bio-imaging systems G-box).

2.5 Lateral flow dipstick (LFD) detection

After amplification, biotin-labeled LAMP products were obtained. DNA-DNA hybridization was accomplished by the addition of the FITC-labeled probe and variation incubation of the mixture at 50-74°C for 5 min. Variation of concentrations of FITC-labeled probe were attained at 0.05, 0.1 and 1.0 µM. Then, the reaction mixture was transferred to a buffer solution before placement of the commercial LFD strip (Milenia® GenLine HybriDetect, Germany) into the mixture tube. Through capillary force, the mixture moved to the conjugated pad, where FITC at the 5' end was captured by a rabbit anti-FITC antibody coupled with AuNPs and bound to the end of the membrane. The result was visualized by the naked eye within 5 min.

2.6 Gold nanoparticles (AuNPs) assay

2.6.1 Synthesis of AuNPs

In preparation of the AuNP solution, the containers were cleaned with aqua regia solution ($HCl+HNO_3$ = 3:1) and Milli-Q water. Briefly, 250 ml of 1 mM hydrogen tetrachloroaurate(III) trihydrate ($HAuCl_4.3H_2O$) was dissolved in Milli-Q water (0.09845 g Au + 250 ml H_2O). Then, the gold solution was transferred into a flask and boiled with a stirrer until the gold solution became vigorous. Simultaneously, 25 mL of 38.8 mM sodium citrate tribasic dehydrate($HOC(COONa)(CH_2COONa)$ $2.2H_2O$) was dissolved with water (0.2852 g sodium citrate + 25 ml H_2O). When the gold solution was refluxed vigorously, sodium citrate solution was quickly added, and refluxing was continued for 15 min until the point at which the color of the solution changed from yellow to clear, black, purple and, finally, deep red. The solution was refluxed for another 15 min before cooling the solution to room temperature (2-4 hr). The final AuNPs were 10-50 nm in diameter with an LSPR of 520 nm. AuNPs solution was stored at 4°C in the dark condition.

2.6.2 Preparation of the AuNPs-labeled thiol probe

The thiol-labeled probe was conjugated with AuNPs by the addition of 4 mL of colloidal AuNPs into a 15-ml centrifuge tube with 20 µL of 100 µM thiol-labeled DNA probe. Then, the bottle was wrapped in aluminum foil and shaken at 138g at 50°C for 24 hr. Meanwhile, washing buffer (10 mM phosphate buffer, pH 7.0, 0.01% SDS or surfactant solution and 100 mM NaCl or salting buffer) was prepared and transferred into the mixture tube. At this step, the color of the solution mixture remained reddish, and no aggregates of AuNPs were observed. The tube was wrapped in aluminum foil and shaken again at 138g at 50°C for 48 hr. After that, the AuNPs-labeled probe in solution was divided into clean 1.5-mL microcentrifuge tubes and centrifuged at 20,630g at 4°C for 30 min. Then, the supernatants were removed. The pellet was washed twice with 500 µL of washing buffer and centrifuged at 20,630g at 4°C for 30 min. Finally, 100-200 µL of washing buffer was added to the AuNPs-labeled DNA probe and stored at 4°C (not frozen) until use. The spectral properties of the AuNPs-labeled DNA probe solution were determined using a UV-VIS spectro-photometer.

2.6.3 LAMP-AuNPs

After LAMP amplification, the AuNP-labeled thiol DNA probe was hybridized with LAMP products at 54-70°C for 5 min (ratio of 1:1). After hybridization, variation of $MgSO_4$ concentration in the presence of 0.01-2.0 M (the ratio of AuNP-labeled thiol DNA probe: LAMP product: $MgSO_4$ was 1: 1: 1), the results were directly examined by the naked eye based on the change in the solution color and were

confirmed by UV-VIS spectral analysis (NanoDrop 2000).

2.6.4 LAMP-real-time turbidimetry

The variations of $MgSO_4$ concentration for LAMP-real-time turbidimetry were 2.4-12.0 mM, determined using a Real-Amp analyzer at 63°C (Mobilis Automata®, Thailand). The formation of magnesium pyrophosphate precipitates was detected at 650 nm.

2.7 Analytical sensitivity and specificity tests

The analytical sensitivity of the PCR and LAMP-based assays was measured by using ten-fold serial dilutions of extracted genomic DNA from 1.24×10^8-1.24 copies/mL. The analytical specificity of all assays was tested by measuring cross-reactivity with HCV, human plasma and a negative control sample (without DNA).

2.8 Validity and predictive values based on unknown samples

One hundred unknown HBV included positive and HBV negative blood samples (positive was sample from infected HBV person and negative was sample from non-infected HBV person); all samples were confirmed using the HBsAg serology test and real-time PCR.

HBV DNA was extracted and isolated according to the manufacturer's protocol using QIAamp DNA kits (Qiagen®, Germany).

The unknown samples were explored using PCR-AGE, LAMP-AGE, LAMP-LFD, LAMP-AuNPs, and LAMP-real-time turbidimetry. The diagnostic sensitivity, diagnostic specificity, accuracy, positive predictive value (PPV) and negative predictive value (NPV) were calculated relative to real-time PCR using 2 x 2 cross-tabulated diagnostic tests. Diagnostic sensitivity: (TP/TP+FN) x 100. Diagnostic specificity: (TN/FP+TN) x 100. Accuracy: [(TP+TN) / Total] x 100. Positive predictive value (PPV): (TP/TP+FP) x 100. Negative predictive value (NPV): (TN/FN+TN) x 100 (Table 1).

Table 1. The 2 x 2 cross-tabulation for the diagnostic tests (HBV DNA positive meaning is the HBV positive by real-time PCR).

Test	Number of sample of each techniques were relatively calculated to "Gold standard (Real-time PCR)"	
	HBV DNA positive	HBV DNA negative
Positive	True Positive (TP)	False Positive (FP)
Negative	False Negative (TN)	True Negative (FN)

3. Results and Discussion

3.1 Optimization of LAMP amplification

3.1.1 Variations of $MgSO_4$ concentrations

The variations of $MgSO_4$ at 1.6-8.0 mM that were carried out at 4.0-6.4 mM yielded the maximum amplification (Fig 1).

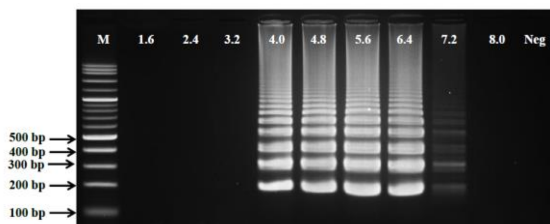

Fig. 1. "M" represents 100 bp plus DNA ladder "Neg" represents negative control.

3.1.2 Variations of temperatures

The variations at 60-65°C that were carried out at 63°C yielded the maximum amplification (Fig 2).

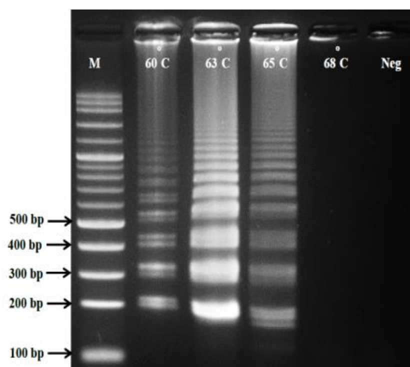

Fig. 2. “M” represents 100 bp plus DNA ladder “Neg” represents negative control.

3.2 Optimization of LAMP-LFD

3.2.1 Variations of temperatures for hybridization

The variations of temperatures for hybridization that were carried out at 54-70ºC explicated the clear color band (Fig 3).

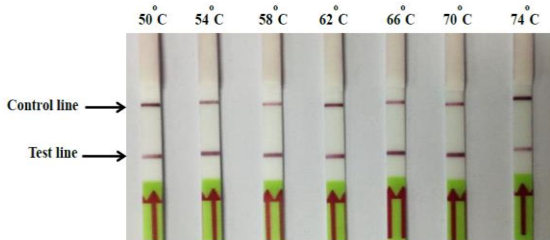

Fig. 3. The variations of temperatures.

3.2.2 Variations of FITC-labeled probe concentrations

The variations of FITC-labeled probe concentrations that were carried out at 0.1µM generated the most intense color band (Fig 4)

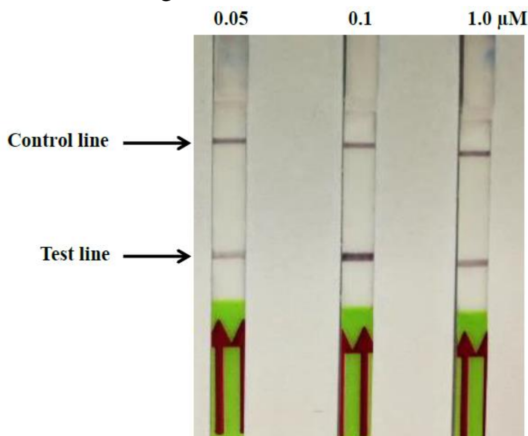

Fig. 4. The variations of FITC-labeled probe concentrations.

3.3 Optimization of LAMP-AuNPs

The variations of $MgSO_4$ concentration for LAMP- AuNPs were carried out at 0.07-2.0 M and showed the distinguished colors for positive and negative solutions (Fig 5).

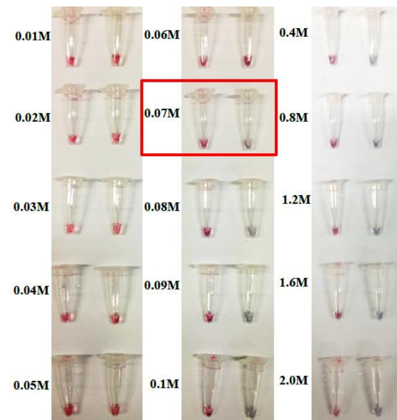

Fig. 5. The variations of $MgSO_4$ concentration**.**

3.4 Optimization of LAMP-real-time turbidimetry

The variations of $MgSO_4$ concentration for LAMP-real-time turbidimetry were carried out at 4.8-12.0 mM and possible signals of positive determined by using turbidimeter (Fig 6).

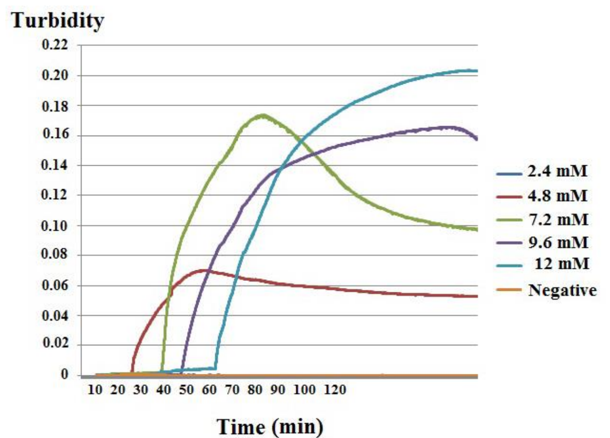

Fig. 6. “Neg” represents negative control.

3.5 Limit of detection (LOD)

3.5.1 Analytical sensitivity

The analytical sensitivities of PCR-AGE (Fig 7a), LAMP-AGE (Fig 7b), LAMP-LFD (Fig 7c), LAMP-AuNPs (Fig 7d) and LAMP-real-time turbidimetry (Fig 7e) were $1.24x10^4$, $1.24x10^1$, $1.24x10^1$, $1.24x10^1$ and $1.24x10^2$ copies/mL, respectively.

3.5.2 Analytical specificity

The analytical specificities were PCR-AGE (Fig 8a), LAMP-AGE (Fig 8b), LAMP-LFD (Fig 8c), LAMP-AuNPs (Fig 8d) and LAMP-real-time turbidimetry (Fig 8e), respectively. The data indicated that the all techniques demonstrated no cross-reactivity with HCV or human plasma.

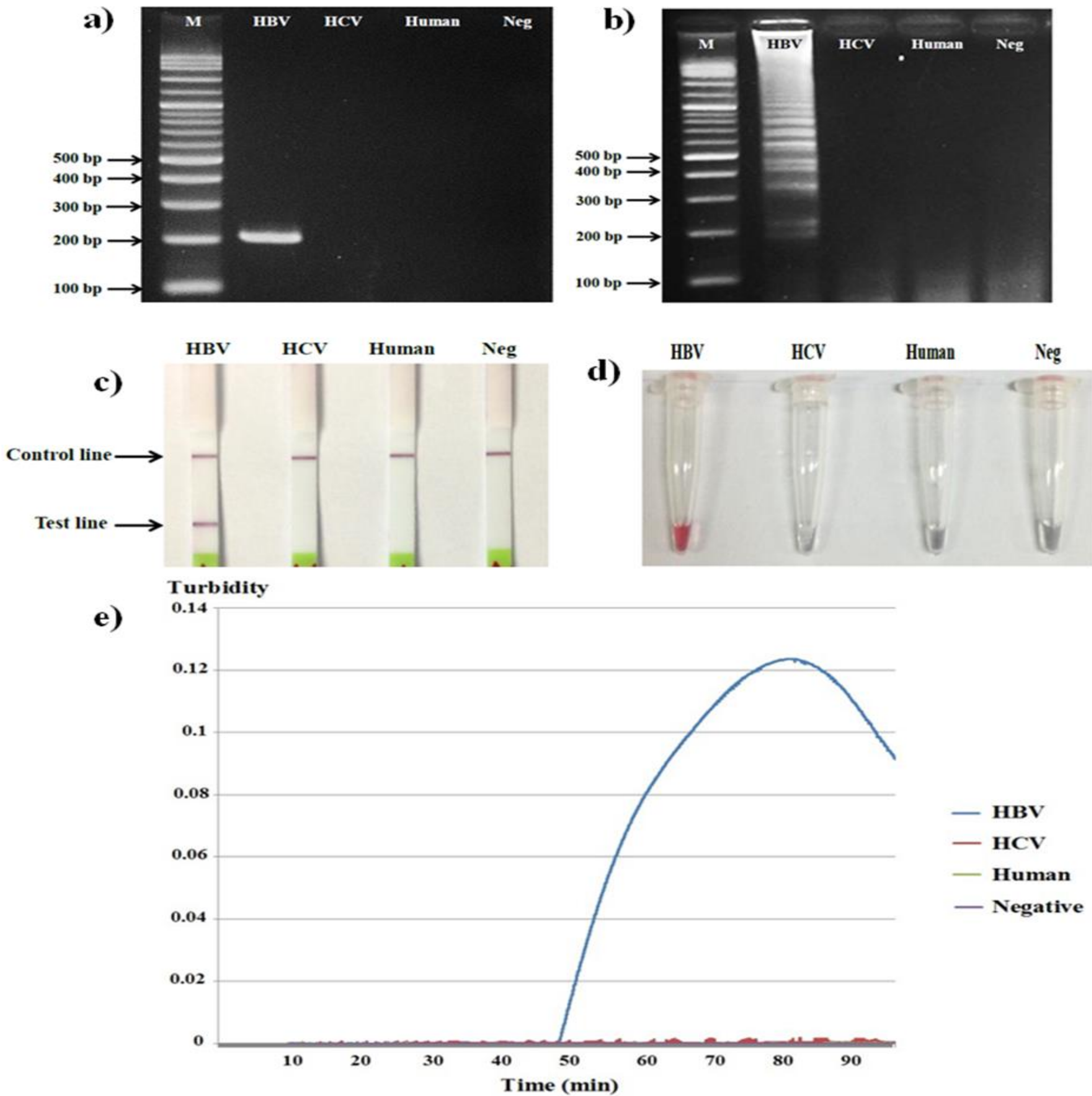

Fig. 7. "M" represents 100 bp plus DNA ladder "Neg" represents negative control.

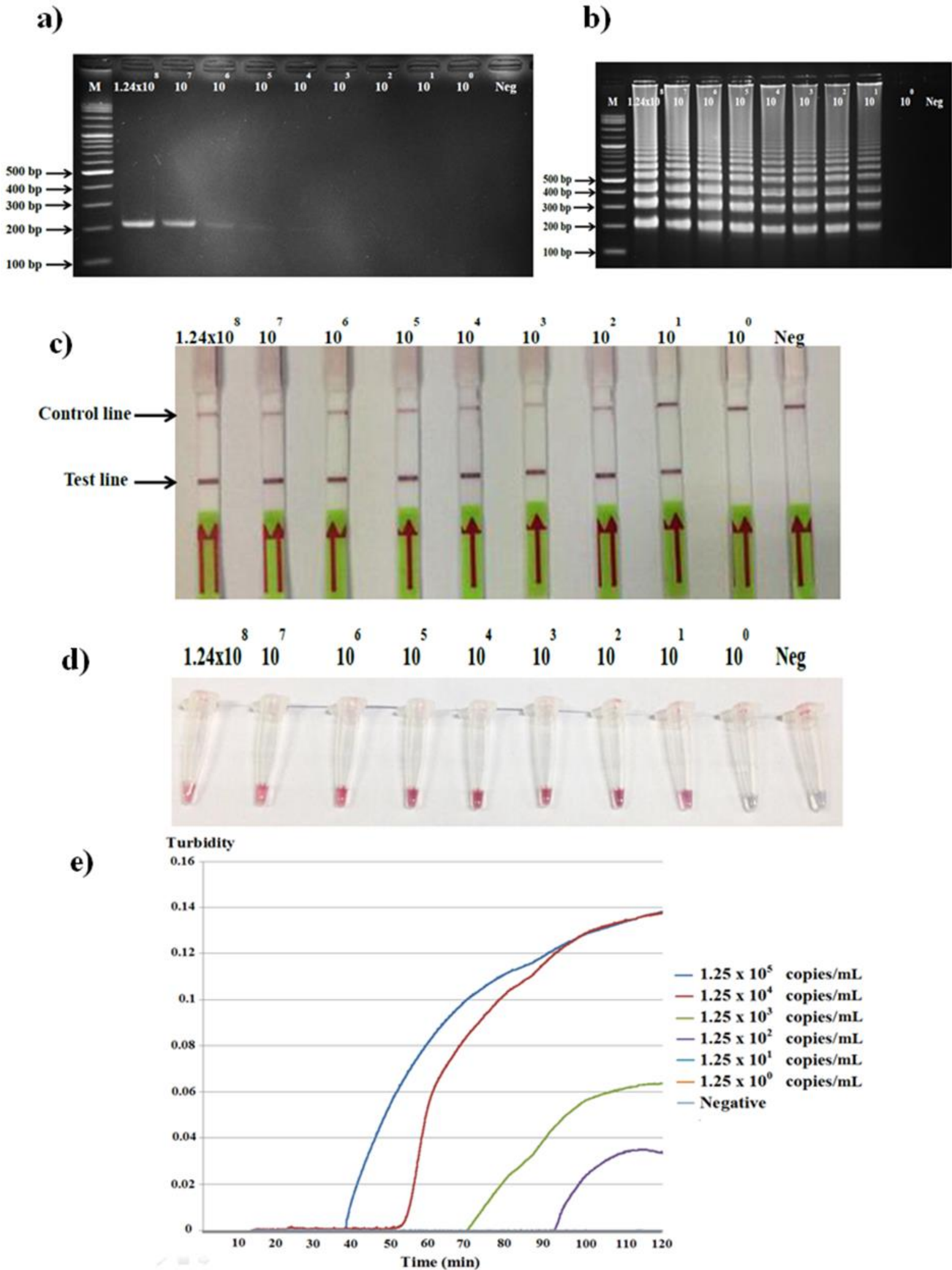

Fig. 8. "M" represents 100 bp plus DNA ladder. "HCV" represents cDNA of HCV. "Human" represents human DNA. "Neg" represents negative control.

3.6 Validity and predictive values based on unknown samples

Of one hundred unknown HBV samples, 80 were HBV positive based on the real-time PCR, while 48 and 82 were HBV positive based on the PCR-AGE and LAMP-based assays, respectively (Table 2).

The diagnostic sensitivity and specificity of all LAMP-based assays were 100% and 90%, respectively. The positive predictive value (PPV) of all LAMP-based assays was 97.56%, while the negative predictive value (NPV) was 100% (Table 3).

Table 2. The HBV screening data of 100 unknown samples were investigated using conventional PCR and LAMP-based assays in comparison to real-time PCR.

Test	Real-time PCR	PCR-AGE	LAMP-based assays			
			AGE	LFD	AuNPs	Real-time turbidimetry
Positive	80	48	82	82	82	82
Negative	20	52	18	18	18	18
Total	100	100	100	100	100	100

Table 3. Validity and predictive values of 100 unknown samples were investigated using conventional PCR and LAMP-based assays in comparison to real-time PCR.

Validity and predicative evaluation	PCR-AGE	LAMP			
		AGE	LFD	AuNPs	Real-time turbidimetry
Sensitivity	60%	100%	100%	100%	100%
Specificity	100%	90%	90%	90%	90%
Accuracy	68%	98%	98%	98%	98%
Positive predictive value	100%	97.56%	97.56%	97.56%	97.56%
Negative predictive value	38%	100%	100%	100%	100%

In this study, LAMP assay combined with LFD, AuNPs and real-time turbidimetry were identified as a set of rapid screening tests for diagnosis of HBV infection.

LAMP-based assays have been developed as rapid screening tests for HBV. The methods are convenient and could be completed within approximately 60 min. The analytical sensitivity of LAMP-LFD and LAMP-AuNPs was 12.4 copies/mL, while that of LAMP-real-time turbidimetry was 124 copies/mL. This result indicated that LAMP-LFD and LAMP-AuNPs were 10 and 1,000 times more sensitive than LAMP-real-time turbidimetry and PCR-AGE, respectively, and could be a proper replacement for AGE in future.

The diagnostic sensitivity of all LAMP-based assays of 100% indicates very high sensitivity, while the diagnostic specificity was 90% and the positive predictive value (PPV) was 97.56%, due to 2 cases showing false positive (negative by gold standard method).

Probably, only 2 cases were shown false positive by LAMP-based assays due to these cases being positive with low virus particles (we have no information for sensitivity of real-time PCR).

Unfortunately, this study did not perform combination tests for co-infection with HBV.

Our preliminary attempt to generate a quantitative assay via LAMP-LFD was achieved using a Quantimeter application on a mobile phone (data not shown). The development of the LAMP-LFD and analysis system is under investigation.

Regarding LAMP-AuNPs, the $MgSO_4$ concentration was the key factor in the maintenance of the solution colors. The suitable concentration of $MgSO_4$ was 0.07-2.0 M, and this solution should always be kept in the dark to avoid the effect of light on the stability of the solution color. In addition to qualification purposes, the quantity of LAMP-AuNP products can be determined using a simple spectro-photometer.

LAMP-real-time turbidimetry was effective for use as a qualitative test, in which the turbidity signal and the reaction time are the key factors for each detection. Nevertheless, false positives may be occasionally occur from any contaminants within the samples that can generate pyrophosphate in the reaction. Notably, the turbidity curve appears to decline over a long period due to the high DNA concentration or viral load in some samples. This effect may involve the excess precipitation of the magnesium pyrophosphate product at the bottom of the tube due to gravity.

4. Conclusion

Among the assays examined in this study, LAMP-LFD and LAMP-AuNPs were capable of use as rapid, sensitive, specific, and easy screening tests that did not require any expensive equipment. However, these assays were essentially qualitative detection methods.

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